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Investigation of Laccase/N-Hydroxybenzotriazole Delignification of Kraft Pulp

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# **INVESTIGATION OF LACCASE/N-HYDROXYBENZOTRIAZOLE DELIGNIFICATION OF KRAFT PULP**

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## **ABSTRACT**

N-hydroxybenzotriazole, a mediator for laccase delignification of kraft pulps, was shown to be unstable under biobleaching conditions. The treatment of N-hydroxybenzotriazole either with laccase alone or in the presence of kraft pulp yielded benzotriazole. The reductive conversion of N-hydroxybenzotriazole to benzotriazole was found to occur rapidly in the presence of pulp. Furthermore, benzotriazole was found to be inactive as a mediator for laccase catalyzed delignification of kraft pulps. Hence, the overall conversion of N-hydroxybenzotriazole to benzotriazole is detrimental toward the bio-delignification process.



## INTRODUCTION

Changes in industrial environmental performance issues and the growing demand by consumers for the production of high quality-products with minimal environmental impact have brought profound changes to the pulp and paper industry.<sup>1</sup> Among the many chemical processes involved in the manufacturing of paper, few processes have drawn as much environmental attention as those involved in the bleaching of pulps. Beginning with the detection of polychlorinated dibenzo-p-dioxins and dibenzofurans in bleach plant effluents<sup>2</sup> in 1987, the papermaking industry has been required to address a series of environmental performance issues. The regulation of AOX (Absorbable Organic Halide) and gaseous chlorinated discharges from modern bleach plant operations is now coming under increasing governmental control.

To address these environmental issues, many new bleaching technologies have been studied. Bleaching chemicals such as chlorine dioxide,<sup>3</sup> ozone,<sup>4</sup> and hydrogen peroxide<sup>5</sup> are being rapidly introduced into commercial practice. Other technologies, including activated hydrogen peroxide<sup>6</sup> and biodelignification<sup>7</sup>, continue to be developed in research laboratories. Recently, laccase mediator biobleaching have garnered increasing attention as researchers have improved the delignification properties of this enzymatic process. Although it was known for some time that laccase could degrade lignin-like structures,<sup>8</sup> its application for delignifying kraft pulps was unsuccessful since the enzyme could not diffuse into pulp fibers due to size constraints.<sup>9</sup> The first true breakthrough in this field came about when Bourbonnais and Paice<sup>10</sup> demonstrated that the addition of ABTS (2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulphonic acid) to laccase resulted in substantial delignification of kraft pulps after an alkaline extraction stage. Representative delignification data acquired in our laboratories<sup>11</sup> for laccase and laccase/ABTS are summarized in Figure 1.

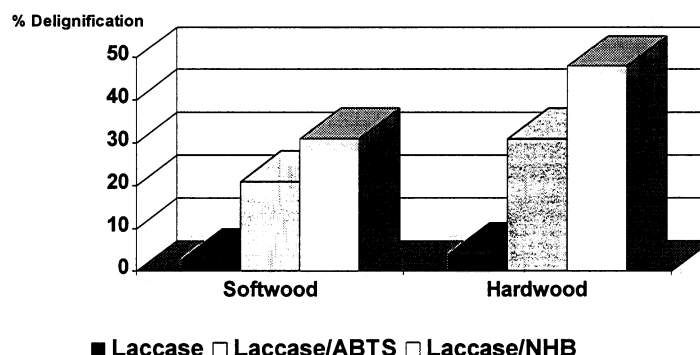


FIGURE 1. Delignification of softwood and hardwood kraft pulps by laccase, laccase/ABTS, and laccase/N-hydroxybenzotriazole.

This discovery initiated a flurry of fundamental research studies into the mechanism of ABTS/laccase bleaching.<sup>12</sup> The results of these studies led to the suggestion that the mechanism of delignification for laccase/ABTS is based on a series of connective oxidative reactions, as summarized in Figure 2. The exact role of ABTS in the delignification process remains controversial, as Potthast et al.<sup>13</sup> and Fiechter et al.<sup>14</sup> have suggested that the laccase-ABTS system does not generate a radical cation, and recently, Paice<sup>16</sup> proposed that the true delignification agent is the dication of ABTS.

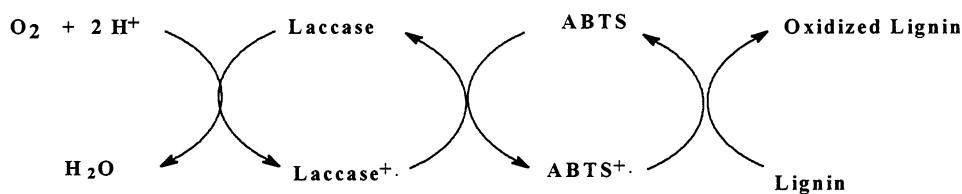


FIGURE 2. Proposed laccase/ABTS delignification mechanism.

Recently, Call<sup>15</sup> identified a new mediator, N-hydroxybenzotriazole, that exhibited improved bleaching performance. Figure 5 provides a comparative

illustration of the bleaching capabilities of ABTS and N-hydroxybenzotriazole with laccase followed by an alkaline extraction stage. Since this initial discovery, our research group<sup>17</sup> and others<sup>18,19,20</sup> have been actively involved in studying the fundamental biobleaching principles involved in this delignification system. Recently, we determined that N-hydroxybenzotriazole was not stable under the bleaching conditions employed, and this report summarizes our investigations into this phenomenon.

## RESULTS AND DISCUSSION

Based on literature, the true delignification agent for a laccase/mediator bleaching system is the oxidized form of the mediator. Hence, in a series of preliminary <sup>1</sup>H NMR experiments, we attempted to detect the active form of N-hydroxybenzotriazole when reacted with laccase in D<sub>2</sub>O. As shown in Figure 1, it was apparent that the enzymatic treatment was converting N-hydroxybenzotriazole to a new material (see Figure 3).

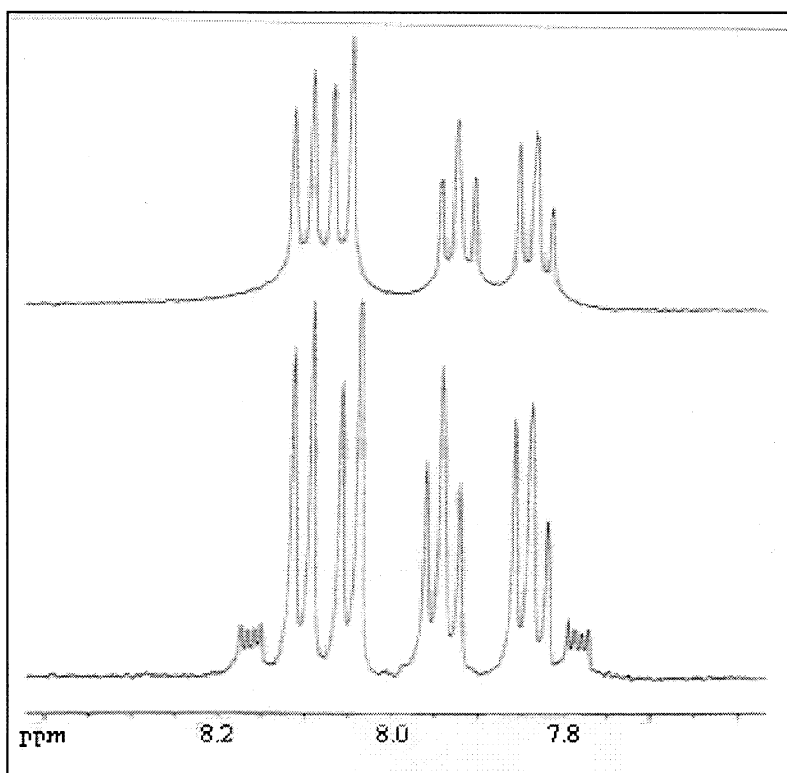


FIGURE 3. <sup>1</sup>H NMR spectra of N-hydroxybenzotriazole in D<sub>2</sub>O (Top) and <sup>1</sup>H NMR spectra of the reaction between laccase (5.2 U) and N-hydroxybenzotriazole for 4 hours at 23°C (Bottom).

Preparative TLC chromatography allowed for isolation of some of the converted material from two separate large-scale reactions between laccase and N-hydroxybenzotriazole. The new compound was characterized by NMR (<sup>1</sup>H and <sup>13</sup>C) and mass spectroscopy (EI and exact mass) analysis, and all analyses indicated that this new component was benzotriazole. The other compound that was isolated (characterized by <sup>1</sup>H and <sup>13</sup>C NMR) was N-hydroxybenzotriazole. This previously unreported reaction suggested that under our conditions N-hydroxybenzo-triazole was converted to benzotriazole.

The conversion of N-hydroxybenzotriazole to benzotriazole was monitored by <sup>1</sup>H NMR, and these results are summarized in Figure 4.



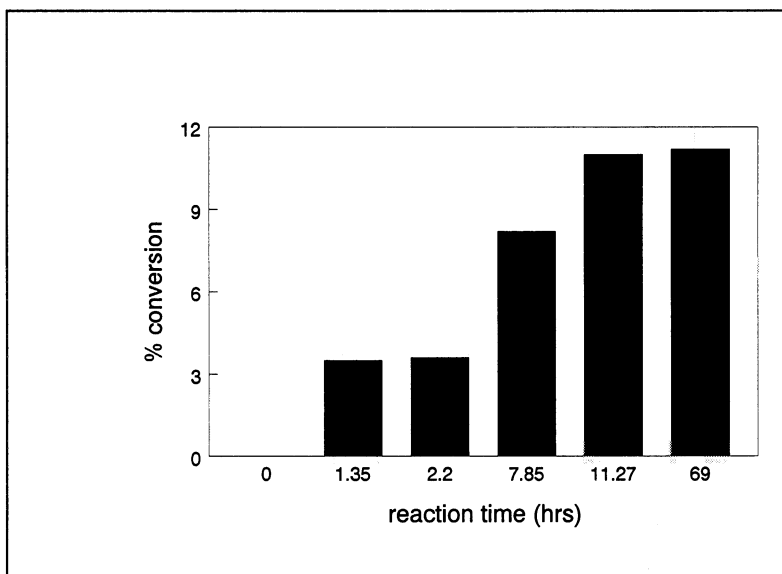


FIGURE 4.  $^1\text{H}$  NMR monitored reaction of N-hydroxybenzotriazole and laccase at  $45^\circ\text{C}$ . Percent conversion was calculated from the ratio of the integration of N-hydroxybenzotriazole and the new species.

The time studies demonstrate that laccase was capable of converting 11% of N-hydroxybenzotriazole to benzotriazole. Several control experiments were performed whereby N-hydroxybenzotriazole was heated at  $45^\circ\text{C}$  with and without oxygen for 24 hour and no converted product was detected. N-hydroxybenzotriazole was also reacted with denatured laccase, and no converted product was detected. It was therefore concluded that the conversion process is initiated by the active form of laccase.

To determine if this process was relevant to the laccase/N-hydroxybenzotriazole biobleaching system, we examined the fate of the mediator at the conclusion of a laccase mediator stage (LMS). A soxhlet-extracted brownstock kraft pulp was treated with the LMS-stage for 24 hours. After the LMS-stage, the pulp mixture was washed with distilled water, air dried, and soxhlet extracted with acetone. Analysis of the soxhlet extracts, which represented 34% of the total

weight of mediator, by  $^1\text{H}$  NMR indicated a 16:84 mixture of N-hydroxybenzotriazole to benzotriazole.

The water washing was freeze dried and soxhlet extracted with acetone and then analyzed by  $^1\text{H}$  NMR. Analysis of the acetone extracts, which represented 65% of the total weight of mediator, indicated the presence of only N-hydroxybenzotriazole and benzotriazole in a 16:84 mixture. The combined yield from the pulp and effluents was 99%, suggesting that the conversion of the mediator to benzotriazole was a dominant reaction during the LMS-stage.

To explore the relevancy of this mediator conversion pathway to the biobleaching process, we repeated the LMS-bleaching stage with benzotriazole. After 24 hours, no decrease in kappa number was observed after the LMS-stage and subsequent extraction. In contrast, if N-hydroxybenzotriazole was employed as the mediator, the kappa number of the pulp decreased from 26.8 to 18.5 after an LMS-stage followed by an alkaline extraction. Hence, the conversion of N-hydroxybenzotriazole to benzotriazole must be viewed as a deleterious pathway that reduces the efficiency of a LMS-stage.

The conversion of N-hydroxybenzotriazole to benzotriazole was further examined by LMS treating an oxygen delignified softwood kraft pulp. The ratio of N-hydroxybenzotriazole:benzotriazole in the LMS effluent was monitored by proton NMR, and the extent of delignification was determine after the pulps were alkaline extracted. The results of these investigations are shown in Figure 5. LMS treatment of the post- $\text{O}_2$  kraft pulp showed significant delignification occurred within the first 5 hours.

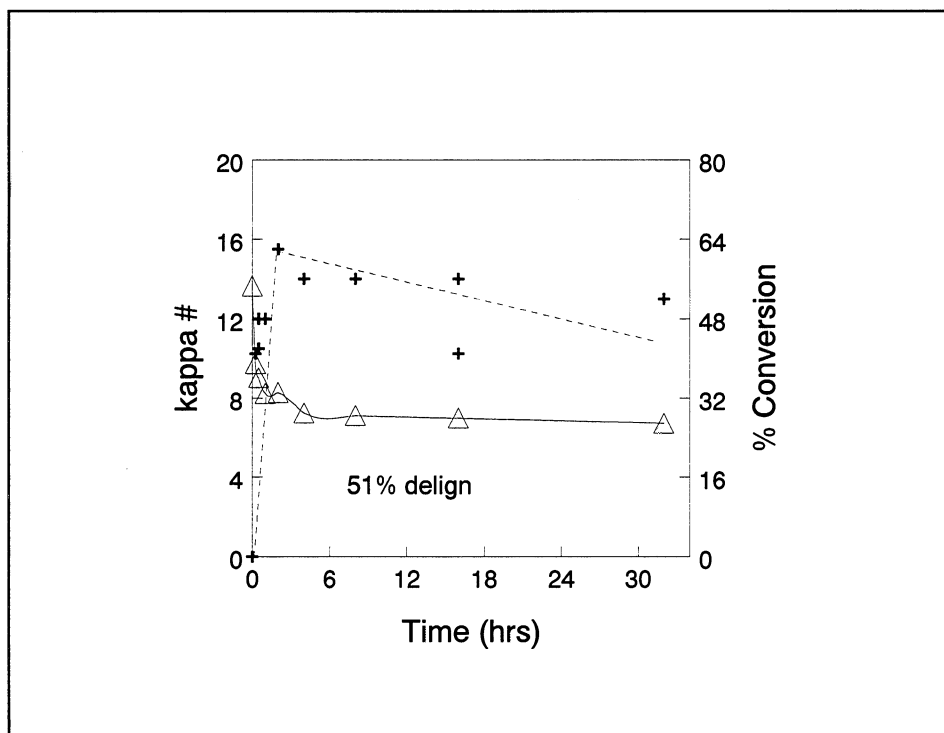


FIGURE 5. Kappa number ( $\Delta$ ) and percent conversion of N-hydroxybenzotriazole to benzotriazole (+) vs. reaction time for an oxygen delignified softwood kraft pulp. All reactions were performed under 145 psi  $O_2$  with a 2% N-Hydroxybenzotriazole charge and  $1.7 \times 10^6$  U of laccase per 10 g of OD pulp.

The conversion of N-hydroxybenzotriazole to benzotriazole occurred rapidly with more than 40% conversion of the mediator after 15 minutes of treatment. The rate of conversion of N-hydroxybenzotriazole to benzotriazole appeared to correlate with the overall trends in delignification.

The conversion of N-hydroxybenzotriazole to benzotriazole under LMS conditions was recently reported by Potthast et al.,<sup>22</sup> Sealey and Raguaskas,<sup>23</sup> and Paice et al.<sup>24</sup> Studies by Potthast with model compounds led to the suggestion that the conversion of N-hydroxybenzotriazole to benzotriazole occurs via a radical coupling process between a phenolic radical and the radical intermediate of

NHB, followed by a subsequent rearrangement that yields benzotriazole as shown in Figure 6.

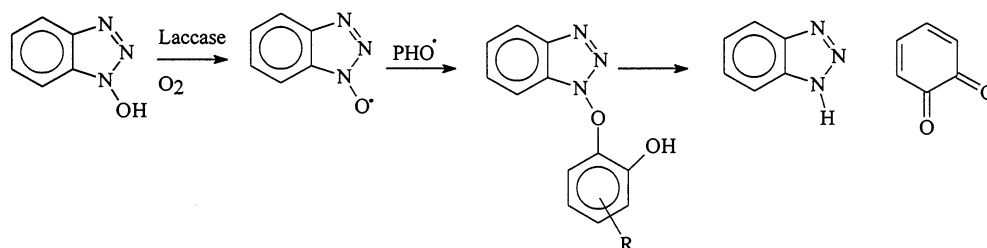


FIGURE 6. Potthast et al.<sup>22</sup> proposed mechanism of benzotriazole generation from N-hydroxybenzotriazole during a LMS treatment.

This reaction mechanism accounts for the observed mediator conversion for the LMS-stage. For the initial NMR studies, the reductive conversion could potentially involve the N-hydroxybenzotriazole radical attacking the enzyme.

## CONCLUSIONS

These studies document the conversion of N-hydroxybenzotriazole to benzotriazole. Clearly, the mediator is not stable under the biobleaching conditions employed, and its conversion to an inactive form is detrimental to the delignification of kraft pulps. It is interesting to note that despite the instability of N-hydroxybenzotriazole under the biobleaching conditions employed, substantial delignification of kraft pulps is still feasible. Clearly, the future research challenge for LMS delignification is the need to find true catalytic mediators.

## EXPERIMENTAL

### Materials and Methods

N-hydroxybenzotriazole, benzotriazole, 1.00 N HCl, tetramethylsilane (TMS), methylene chloride, acetone-d<sub>6</sub>, 99.99% D<sub>2</sub>O, and preparative 1000 µm silica gel plates were commercially purchased and used as received. Biobleaching studies employed an industrial, never-dried, softwood kraft pulp (kappa #: 26.8) and an oxygen delignified softwood kraft pulp (kraft #: 13.6). Prior to using the pulps, they were thoroughly washed with deionized water until the washings were pH neutral and colorless.

Laccase, isolated from a *Polyporus* fungi, was provided by Novo Nordisk. The enzyme was frozen to -20°C until use. Once thawed, the activity of the enzyme was measured, and the proper dose was added to the pulp.

### Laccase Assay

The activity of the laccase was measured by monitoring the rate of oxidation of syringaldazine. The change in A<sub>530nm</sub> of 0.001 per minute per mL of enzyme solution in a 100 mM potassium phosphate buffer (2.20 mL) and 0.216 mM syringaldazine in methanol (0.3 mL) was set to one Unit of activity. This test was performed at 23°C.

## Physical and Chemical Characterization of Kraft Pulps

The lignin content of the kraft pulps was determined following standard TAPPI method<sup>13</sup> T-236 and expressed as a “kappa number.”

## <sup>1</sup>H NMR Studies

All NMR studies were performed using a Bruker 400 MHz DMX spectrometer. Typically, proton NMR were acquired with 16-32 scans per spectrum using a 30° pulse. Experiments were recorded at 25° and 45°C. The FID was Fourier Transformed with one degree of zero-filling and 0.30 Hz line-broadening.

## Laccase/N-hydroxybenzotriazole Studies

A sample of N-hydroxybenzotriazole (2 mgr.,  $0.015 \times 10^{-3}$  mols) was dissolved in 0.50 ml of D<sub>2</sub>O. A spectrum of this material was recorded, and 5.20 U of laccase were added (Note: the chemical shifts of N-hydroxybenzotriazole were referenced to TMS). Spectra were recorded after 4 hours of reaction at 25°C (see Figure 3). This reaction was then repeated on a 50-fold larger scale, and the product mixture was freeze dried, placed on a preparative silica gel plate, and developed with methylene chloride. One new product was observed, and isolation of this material indicated that the new material was benzotriazole. All spectral data agreed with literature values:

<sup>1</sup>H NMR(acetone-d<sub>6</sub>):  $\delta$ 7.58 (2H, dd, J=6.0, 3.3 Hz), 8.04 (2H, dd J=6.0, 3.3 Hz)

<sup>13</sup>C NMR (Acetone-d<sub>6</sub>):  $\delta$ 115.0, 125.5, 139.0

EI/MS m/z(rel. intensity): 119(100), 91(90), 64(60), 52(18), 40(10).

HRMS calcd for  $C_6H_5N_3$ : 119.0483, Found 119.0488.

#### Laccase/N-hydroxybenzotriazole Conversion Studies

The conversion of N-hydroxybenzotriazole to benzotriazole in the presence of laccase was monitored over time by  $^1H$  NMR. Conveniently, the unsaturated protons of these two species both appear in the range of 7.70-8.20 ppm and do not overlap, providing a facile means of determining product mixtures. Following the procedure described above, the laccase/N-hydroxybenzotriazole system was maintained at 45°C and periodically examined by NMR. The results of these studies are summarized in Figure 4.

Control experiments with thermally denatured laccase yielded only benzotriazole. Likewise, if the laccase was omitted from the above experiments, only benzotriazole was detected.

#### Laccase/N-hydroxybenzotriazole Biobleaching

Biobleaching for 24 hours: A 10% consistency pulp slurry containing 5.0 gr of fiber (Note: the pulp was acetone extracted prior to use), was added to a preheated pressure vessel maintained at 45°C. To this mixture was added the N-hydroxybenzotriazole (0.10 gr,  $6.74 \times 10^{-3}$  mols) and the resulting mixture was stirred for 3 minutes. The pH of the mixture was then adjusted to 4.5 with glacial acetic acid, and the laccase containing solution ( $120 \times 10^3$  U) was added. The pressure vessel was then sealed, and the reactants were stirred for 24 hours, at 45°C and 10 barr  $O_2$  pressure. After treatment, the mixture was filtered, and the

pulp fibers were washed with deionized water (2000 ml). The aqueous phase was collected, freeze dried, and acetone soxhlet extracted. The extracts (65 mgr.) were concentrated under reduced pressure, and analyzed by  $^1\text{H}$  NMR (acetone- $\text{d}_6$ ); this spectrum contained a 84:16 ratio of benzotriazole and N-hydroxybenzotriazole.

The pulp fibers were dried and soxhlet extracted with acetone for 24 hours. The extracts were concentrated and analyzed by  $^1\text{H}$  NMR(acetone- $\text{d}_6$ ). NMR analysis indicated that the pulp extracts (34 mgr.) contained a 85:16 ratio of benzotriazole and N-hydroxybenzotriazole. The above experiments were repeated, and the yield and product ratio of benzotriazole:N-hydroxybenzotriazole agreed with the prior results to within 1%.

Biobleaching for Varied Times: The above experiments were subsequently repeated using a softwood post-oxygen delignified kraft pulp employing twice the amount of pulp, laccase, and mediator. For these latter experiments, the pulp was not soxhlet extracted. The biobleaching time was varied from 15 minutes to 44 hours. In each case, the bleached pulp was washed with distilled water and the effluents were filtered, freeze dried, and soxhlet extracted with acetone. The acetone fraction was concentrated and analyzed by  $^1\text{H}$  NMR. Table 1 summarizes the ratio of N-hydroxybenzotriazole to benzotriazole detected by NMR and the mass recovery.

To determine the extent of delignification for each laccase/N-hydroxybenzotriazole biobleaching experiment, a pulp sample was subsequently extracted with caustic. This was accomplished by placing the pulp in a plastic bag at 10% consistency with a 2.0% charge of NaOH. The mixture was then sealed, warmed to  $70^\circ\text{C}$ , and mixed occasionally. After 2 hours, the pulp was removed from the water bath, filtered, washed, and air-dried prior to kappa number determination.



TABLE 1

Mass recovery of mediator materials and ratio of  
N-hydroxybenzo-triazole:benzotriazole in the laccase biobleaching effluent.

Biobleaching time/h	% Mediator material recovered	Ratio <sup>a</sup> of N-hydroxybenzotriazole:benzotriazole
0.25	73	59:41
0.50	74	52:48
1.00	63	52:48
2.00	64	38:62
4.00	59	44:56
8.00	56	44:56
16.00	59 (59) <sup>b</sup>	44:56 (59:41)
32.00	55	48:52

<sup>a</sup>ratio was determined by proton NMR; <sup>b</sup>repeat values.

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